REMARKS

Applicants respectfully request reconsideration of this Request for Continued Examination and the claims as herein amended. Pending claims 1-13 and 15-29 are herein amended and new claims 30-42 are newly presented.

Applicants gratefully acknowledge Examiner Kathleen Kerr's interview of October 21st, 2004. The substance of the discussion is summarized in the Interview Summary made of record in the parent case by Examiner Kerr. After carefully considering the objections and rejections raised in the Final Office Action in the parent case, Applicants respectfully request reconsideration and early allowance of the claims herein presented, representing Applicants' and the attorney's understanding of the points discussed during the interview.

The claims herein presented have support in the specification as filed in U.S. non-provisional application Serial No. 10/067,974, and in U.S. provisional application Serial No. 60/267,183, as detailed below. Thus no new matter being introduced, the claims as herein presented are entitled to the earliest priority date of February 8, 2001, and Applicants respectfully and courteously request granting of this earliest priority in this case under 35 U.S.C. § 119(e).

The amendments to the claims are summarized as follows:

Claim 1 is amended to include the limitations of previously filed claim 25 having support in the as-filed provisional application serial number 60/267,183, filed February 8, 2001.

Claim 2 is amended to include a limitation of sequence identity for diaminopimelate dehydrogenase (ddh) "at least 80% identity to SEQ ID NO: 8" as disclosed in the as-filed specification in paragraph [0074].

Claim 3 is amended to change dependency from claim 1 to claim 2 and to include a description of the function for ORF2. A declaration by Applicant Lhing-Yew Li is included in Appendix A to provide support for this description of the function of ORF2. An added limitation of sequence of "at least 90% identical to SEQ ID NO: 9" is supported in paragraph [0068] of the specification.

Claim 4 is amended to include a limitation of sequence identity for ddh polypeptide having "at least 80% identity to SEQ ID NO: 8" as disclosed in the as-filed specification in paragraph [0074]. Claim 4 is also amended to add a structural limitation to the "at least 25% of the full length of an ORF2 polypeptide" by adding specific SEQ ID NO: 10 as the full length ORF2, and similarly adding SEQ ID NO: 12 as the reference point for "at least 80% identity" limitation of diamino decarboxylase polypeptide. Claim 4 is also amended to include the function for ORF2 having thymidilate synthase activity or 2,3 dihydrodipicolinate N-C6-lyase activity. A declaration by inventor Lhing-Yew Li is included in Appendix A to describe the activity of ORF2.

Claims 7, 9, 10 and 11 have been amended to clarify "genes native to" objected by the Examiner to "genes from a cell of the genus *Corynebacterium*." As stated in the interview summary by Examiner Kerr noted that the "genus is adequately represented by the species in the specification."

Claim 8 has been amended to clarify that the ask/asd operon expresses two polypetides each with a different function.

Claims 12, 13, and 15 have been amended from "at least 95% identical" to "at least 90% identical." Support for this amended in found in the specification paragraph [0068].

Claim 25 is amended to further limit the genes excluded by claim 1, from which it depends.

Claims 26-29 are amended to recite *Corynebacterium glutamicum* as the source for the polynucleotide molecules.

Claims 30-36 are added to claim specific host cells of claims 17 and 20 which have been deposited.

Claims 37-39 are added to claim specific vectors of claim 16 which have been described in the Examples of the specification.

Claim 40 is directed to lysine produced by the host cells of the invention transformed with the polynucleotides of the invention.

Claims 41 and 42 are directed to specific embodiments of ORF2 encoding polynucleotide and ORF2 polypeptides.

Objections to the Specification and Claims

Applicants gratefully acknowledge the Examiner's withdrawal of the objections to the specification, items 4, 5 and 6, and claims, item 7, by virtue of Applicants' amendment previously submitted in the parent case.

Rejections under 35 U.S.C. § 112, Second Paragraph

Applicants gratefully acknowledge the Examiner's withdrawal of the objections to the claims 1-23 and 25, items 8-13, by virtue of Applicants' amendment previously submitted in the parent case.

Claims 20, and 26-29 were previously rejected for indefiniteness under 35 U.S. § 112, second paragraph, for reciting both *Brevibacterium* and *Corynebacterium* in a Markush group . As amended herewith, the claims recite only host cells of the genus *Corynebacterium* rendering this rejection moot. Applicants respectfully request withdrawal of this rejection and allowance of claims 20, and 26-29.

Claim 25 was previously rejected under 35 U.S.C. § 112, second paragraph, as indefinite for use of the term "N-succinylaminoketopimelate transaminase." During the interview, Examiner Kerr indicated that there was no enzyme catalogued in the enzyme database with this catalytic activity and that it may be confused with the enzyme succinyldiaminopimelate transaminase, which is catalogued in the enzyme database. Applicants respectfully submit that the term "N-succinylaminoketopimelate transaminase" adequately describes the catalytic activity of the gene product of the dapC gene of Applicants' invention. Applicant Lhing-Yew Li submits an affidavit that details the catalytic activity of this enzyme as described in the literature and sets forth the definiteness of the claim. Applicants respectfully request withdrawal of this rejection and courteously request allowance of claim 25.

Rejections under 35 U.S.C. § 112, First Paragraph

Claims 4-6, 10-11, 14-15, and 28-29 were rejected under 35 U.S.C. § 112, first paragraph, for alleged failure to comply with the written description requirement. The claims subject of this rejection have been amended to recite the activity for the product of the ORF2 polynucleotide of the invention, making these claims definite in scope. Applicant Lhing-Yew Li makes a declaration herewith appended detailing the enzymatic activities of the ORF2 polypeptide of the instant invention. Applicants respectfully request withdrawal of this rejection and courteously request allowance of claims 4-6, 10-11, 14-15, and 28-29.

Rejection under 35 U.S.C. § 102(a)

Claims 1-13 and 15-23 were rejected under 35 U.S.C. § 102(a) in view of Li *et al.*, WO 01/49854 ("the '854 Application"), which has an international filing date of December 29, 2000, and was published on July 12, 2001. As herein amended, pending claims 1-13, 15-23, 25-42 recite "80% sequence identity to SEQ ID NO: 8" and are supported in the as-filed provisional application serial number 60/267,183 as detailed above, and thus the instant application should be granted priority to its filing date of February 8, 2001. Applicants respectfully request that priority be granted under 35 U.S.C. § 119(e) to all pending claims in the instant application, to the priority date of February 8, 2001, making the cited reference by Li *et al.*, unavailable as prior art reference. Applicants respectfully request that the rejection of claims 1-13, and 15-23 under 35 U.S.C. § 102(a) in view of Li *et al.* be withdrawn. Applicants courteously request allowance of claims 1-13 and 15-23 at an early date.

Rejection under 35 U.S.C. § 102(e)

Claims 1-4, 7-18, and 20-21 were provisionally rejected under 35 U.S.C. § 102(e) in view of Hanke *et al.*, U.S. Application No. 09/722,441 ("the '441 Application"). Claims 1-13, 23-42 as herein amended are distinct from Hanke *et al.* by reciting additional limitations and excluding at least one gene from the dihydrodipicolinate synthase, tetrahydrodipicolinate succinylase, N-succinylaminoketopimelate transaminase, N-succinyl-diaminopimelate desuccinylase, and diaminopimelate epimerase lysine synthesis pathway. Thus, Applicants respectfully request the rejection of claims 1-4, 7-18, and 20-21 under 35 U.S.C § 102(e) in view of Hanke *et al.* be withdrawn and the claims allowed.

35 U.S.C. §1.132 Declaration to Distinguish '441 Application

Attached as Appendix A is a Declaration of Lhing-Yew Li pursuant to 37 C.F.R. § 1.132, describing Applicant Lhing-Yew Li's contributions to the '441 Application as distinct from the instant invention, the function of ORF2 polypeptide of the instant invention, and the activity of the dapC gene product N-succinyl-aminoketopimelate transaminase.

CONCLUSION

It is respectfully submitted that Applicants' Claims 1-13,15-23, and 25-29 as herein amended, and newly presented claims 30-42 illustrate patentable compositions and methods to produce L-Lysine in host cells that are not taught or suggested in the art of record. Applicants respectfully submit that the amendments and remarks set forth in this paper place this Application in condition for allowance and such action is courteously requested at an early date. Prompt and favorable consideration of this Response and Amendment and Request for Continued Examination is respectfully requested.

AUTHORIZATION

The Commissioner is hereby authorized to charge any necessary additional fees associated with this paper to Deposit Account No. 02-4553. A duplicate copy of this Response and Amendment is enclosed for deposit account purposes.

Respectfully submitted,

Buchanan Ingersoll PC

Craig G. Cochenour

Dated: December 7, 2004

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Pittsburgh, Pennsylvania 15219
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APPENDIX A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group Art Unit: 1652

Examiner: Kathleen M. Kerr

In re application of
LHING-YEW LI *et al.*

Serial No.: 10/067,974

Filed: February 8, 2002

PATENT APPLICATION

POLYNUCLEOTIDE CONSTRUCTS ENCODING ASPARTATE KINASE, ASPARTATE-SEMIALDEHYDE DEHYDROGENASE, AND DIHYDRODIPICOLINATE REDUCTASE AND RELATED CONSTRUCTS, PRODUCTS AND METHODS

Group Art Unit: 1652

DECLARATION OF LHING-YEW LI
PURSUANT TO 37 C.F.R. § 1.132

I Lhing-Yew Li, declare as follows:

1. I am an inventor of the isolated polynucleotide molecules which are the subject of the above captioned U.S. patent application.

2. I hold a Ph.D. in Microbiology from the University of Illinois, Urbana-Champaign, and have been working as a research scientist in this field since 1994.

3. I am currently a Program Manager employed by Archer Daniels Midland Company.

4. The ORF2 polynucleotides of the above captioned U.S. patent application encode a polypeptide having predicted function as thymidilate synthase or as 2,3-dihydrodipicolinate N-C6-lyase as described in an abstract by Pisabarro *et al.* for locus B40626, a copy of which is appended herewith.

5. The dapC polynucleotides of the above captioned U.S. patent application encode polypeptides having N-succinyl-aminoketopimelate transaminase activity that is distinct from the activity of the dapE gene product having N-succinyl-diaminopimelate desuccinylase catalogued in the enzyme database. The enzymatic activity of the dapC gene product is described in the publication by Hartmann *et al.* (J. Biotechnol. 2003 Sep. 4; 104 (1-3):199-211) as N-succinyl-aminoketopimelate aminotransferase (transaminase), a copy of which is herewith appended.

6. I am an inventor of United States Patent Application No. 09/722,441 ('441 Application). I am the sole inventor of the conception, design, and construction of the gene cassettes for the expression of the isolated polynucleotides described in the '441 Application.

7. I am an inventor of the isolated polynucleotides of the instant invention useful for the transformation of host cells for the production of lysine, which isolated polynucleotides are introduced into the host cells of the invention using the cassettes described in the '441 Application.

I declare that the foregoing is true and correct, that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any false statement may jeopardize the validity of a patent which issues from the above captioned patent application.

Further I sayeth not.

Date Dec. 6, 2004

Lhing-Yew Li

☐ 1: B40626. Reports probable 2,3-dihy...[gi:2127423] BLink, Domains, Links

```
LOCUS       B40626                    250 aa            linear   BCT 22-OCT-1999
DEFINITION  probable 2,3-dihydrodipicolinate N-C6-lyase (cyclizing) (EC
            4.3.3.-) - Corynebacterium glutamicum.
ACCESSION   B40626
VERSION     B40626  GI:2127423
DBSOURCE    pir: locus B40626;

            summary: #length 250 #molecular-weight 28085 #checksum 7567
            ;
            PIR dates: 21-Sep-1993 #sequence_revision 15-Oct-1996 #text_change
            22-Oct-1999
            .
KEYWORDS    carbon-nitrogen lyase; diaminopimelate-lysine biosynthesis.
SOURCE      Corynebacterium glutamicum
  ORGANISM  Corynebacterium glutamicum
            Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
            Corynebacterineae; Corynebacteriaceae; Corynebacterium.
REFERENCE   1  (residues 1 to 250)
  AUTHORS   Pisabarro,A., Malumbres,M., Mateos,L.M., Oguiza,J.A. and
            Martin,J.F.
  TITLE     A cluster of three genes (dapA, orf2, and dapB) of Brevibacterium
            lactofermentum encodes dihydrodipicolinate synthase,
            dihydrodipicolinate reductase, and a third polypeptide of unknown
            function
  JOURNAL   J. Bacteriol. 175 (9), 2743-2749 (1993)
  MEDLINE   93239702
   PUBMED   8478336
COMMENT     On May 28, 1997 this sequence version replaced gi:423987.
FEATURES             Location/Qualifiers
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                     (cyclizing)"
                     /EC_number="4.3.3.-"
                     /note="cyclization polypeptide"
     Region          13..229
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                     /note="Thy1"
                     /db_xref="CDD:17159"
ORIGIN
        1 maeqvklsve liacssftpp advewstdve gaealvefag racyetfdkp nprtashaay
       61 lrhimevght allehanatm yirgisrsat helvrhrhfs fsqlsqrfvh sgesevvvpt
      121 lidedpqlre lfmhamdesr fafnellnal eeklgdepha llrkkqarqa aravlphate
      181 srivvsghfr twrhfigmra sehadveire vavgclrklq vaaptvfgdf eietladgsq
      241 matspyvmdf
//
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Identification and characterization of the last two unknown genes, *dapC* and *dapF*, in the succinylase branch of the L-lysine biosynthesis of *Corynebacterium glutamicum*

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Received 16 December 2002; received in revised form 31 January 2003; accepted 11 March 2003

Abstract

The inspection of the complete genome sequence of *Corynebacterium glutamicum* ATCC 13032 led to the identification of *dapC* and *dapF*, the last two unknown genes of the succinylase branch of the L-lysine biosynthesis. The deduced DapF protein of *C. glutamicum* is characterized by a two-domain structure and a conserved diaminopimelate (DAP) epimerase signature. Overexpression of *dapF* resulted in an 8-fold increase of the specific epimerase activity. A defined deletion in the *dapF* gene led to a reduced growth of *C. glutamicum* in a medium with excess carbon but limited ammonium availability. The predicted DapC protein of *C. glutamicum* shared 29% identical amino acids with DapC from *Bordetella pertussis*, the only enzymatically characterized *N*-succinyl-aminoketopimelate aminotransferase. Overexpression of the *dapC* gene in *C. glutamicum* resulted in a 9-fold increase of the specific aminotransferase activity. A *C. glutamicum* mutant with deleted *dapC* showed normal growth characteristics with excess carbon and limited ammonium. Even a mutation of the two genes *dapC* and *ddh*, interrupting both branches of the split pathway, could be established in *C. glutamicum*. Overexpression of the *dapF* or the *dapC* gene in an industrial *C. glutamicum* strain resulted in an increased L-lysine production, indicating that both genes might be relevant targets for the development of improved production strains.

Keywords: *Corynebacterium glutamicum*; Lysine biosynthesis; Diaminopimelate biosynthesis; Lysine production

1. Introduction

The bacterial diaminopimelate (DAP) and L-lysine biosynthesis pathway was elucidated by a series of biochemical studies in the 1960s and 70s (reviewed in Patte, 1996). Three alternative path-

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ways leading to the synthesis of D,L-DAP were identified in bacteria (Scapin and Blanchard, 1998). They share the first steps of aspartate (and pyruvate) conversion to the intermediate L-2,3,4,5-tetrahydrodipicolinate (THDP), but vary in the subsequent reactions (Fig. 1). THDP can be converted to DAP in a single reaction which is catalyzed by an ammonium-incorporating diaminopimelate dehydrogenase, whereas the other pathway variants consist of four reaction steps involving either acetylated or succinylated intermediates. The dehydrogenase and/or acetylase variants of the L-lysine biosynthesis were found among the members of the genus *Bacillus* (Weinberger and Gilvarg, 1970), whereas the succinylase pathway is present in *Escherichia coli* and most other bacteria (Kindler and Gilvarg, 1960; Scapin and Blanchard, 1998). In the succinylase pathway, THDP is succinylated to *N*-succinyl-2-amino-6-ketopimelate which is the substrate of the aminotransferase DapC. The product of this reaction is then desuccinylated to give L,L-DAP which is converted by the epimerase DapF to the penultimate L-lysine precursor D,L-DAP (Fig. 1).

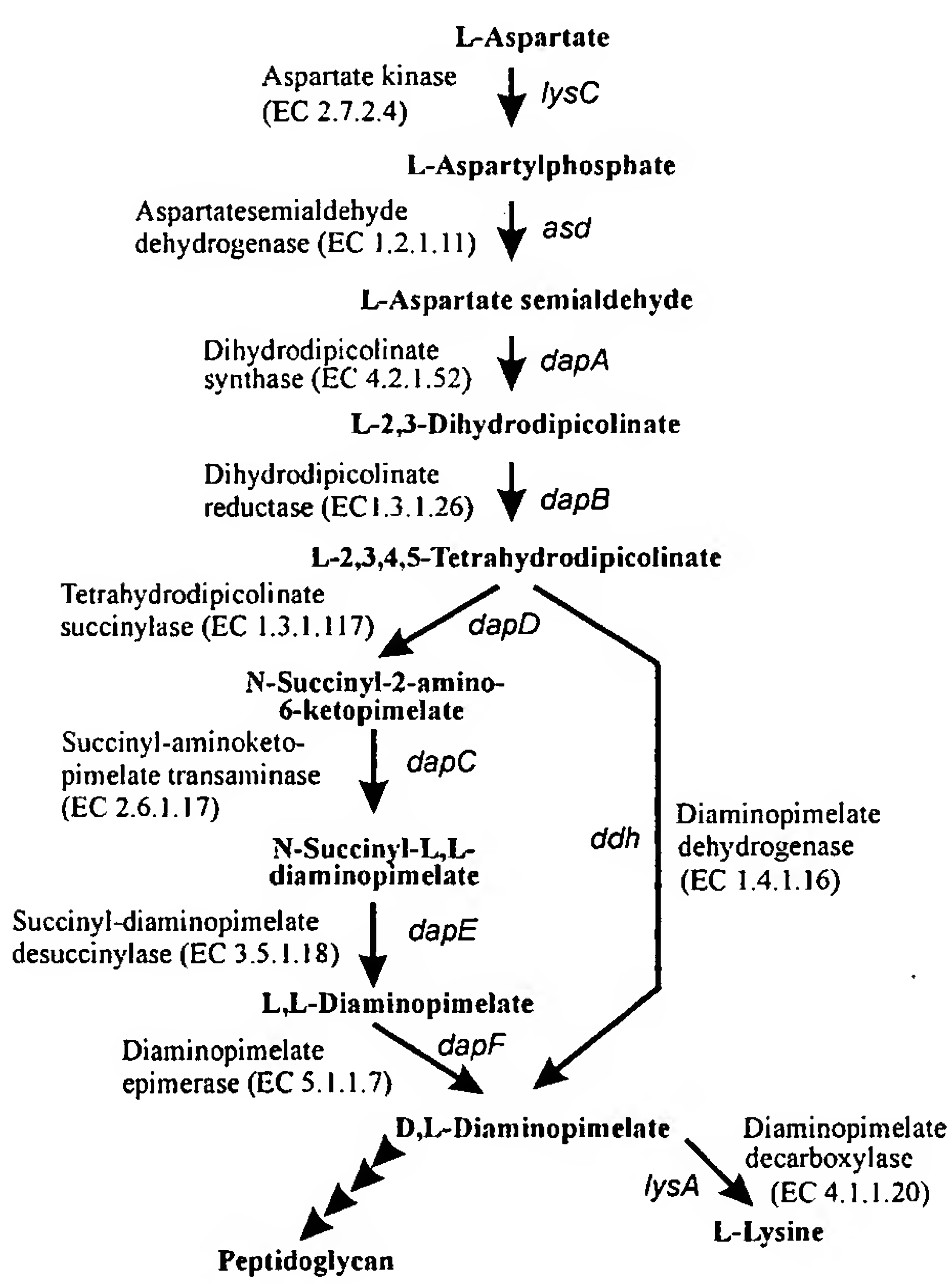

Fig. 1. The branched pathway for D,L-diaminopimelate and L-lysine biosynthesis in *C. glutamicum*.

As a special feature, the gram-positive soil bacterium *Corynebacterium glutamicum* possesses the succinylase together with the dehydrogenase variant of the D,L-DAP and L-lysine biosynthesis (Schrumpf et al., 1991). Both branches operate in parallel in this organism as shown by metabolite flux analyses (Sonntag et al., 1993). Currently, more than 6.0×10^5 tons of L-lysine are produced annually with *C. glutamicum* mutant strains. Therefore, tremendous efforts are constantly undertaken to optimize the L-lysine biosynthesis with regard to higher efficiencies of such strains (Leuchtenberger, 1996). Genes directly involved in the synthesis of L-lysine are obviously primary targets to improve the overall fermentation process. Most of these genes were already identified in *C. glutamicum* (Cremer et al., 1990; Ishino et al., 1987; Kalinowski et al., 1990, 1991; Wehrmann et al., 1994, 1998; Yeh et al., 1988). However, *dapC* and *dapF* encoding *N*-succinyl-aminoketopimelate aminotransferase and diaminopimelate epimerase still remain to be identified (Fig. 1).

The aminotransferase DapC (EC 2.6.1.17) catalyzes the transfer of the amino group from glutamate to *N*-succinyl-L,L-diaminopimelate forming α-ketoglutarate and *N*-*N*-succinyl-2,6-L,L-diaminopimelate. Like all known aminotransferases it uses pyridoxal 5′-phosphate (PLP) as a catalytic cofactor. The DapC enzyme of *Bordetella pertussis* (Fuchs et al., 2000) and the ArgD protein of *E. coli* are the sole examples of bacterial aminotransferases with this substrate specificity as disclosed in protein databases (Ledwidge and Blanchard, 1999). The ArgD protein of *E. coli* possesses both an acetylornithine and a DAP aminotransferase activity, explaining its nomination as ArgD. Interestingly, the *B. pertussis* DapC protein and ArgD of *E. coli* share no significant amino acid sequence similarity apart from the characteristic PLP-binding domain (Fuchs et al., 2000). Despite the availability of a large number of whole genome sequences from bacteria, no other gene encoding an *N*-succinyl-L,L-DAP amino-

transferase has been characterized enzymatically so far.

In the last step of the succinylase branch, D,L-DAP is generated from the corresponding L,L-isomer by the *dapF*-encoded diaminopimelate epimerase (EC 5.1.1.7). The DapF protein is a representative of a pyridoxal phosphate-independent amino acid racemace (Koo and Blanchard, 1999). DapF of *E. coli* was purified and characterized (Wiseman and Nichols, 1984) and the corresponding gene was cloned and mapped in the *E. coli* chromosome (Richaud et al., 1987; Richaud and Printz, 1988). Currently, at least 25 homologous protein sequences of other organisms have been deposited in protein databases, all containing a specific diaminopimelate epimerase signature (PROSITE PDOC01029), which clearly defines this protein family. Herein included are the corresponding DapF proteins of *Mycobacterium tuberculosis* and *Streptomyces coelicolor* which are close taxonomic relatives of *C. glutamicum*.

In the present paper, we report how the *C. glutamicum* genome sequencing project enabled us to identify and to characterize the diaminopimelate epimerase gene *dapF* and the *N*-succinylaminoketopimelate aminotransferase gene *dapC*. Both genes showed significant effects on L-lysine production when overexpressed in an industrial *C. glutamicum* strain.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Relevant bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *C. glutamicum* strains were routinely grown in Luria-Bertani medium (Sambrook et al., 1989) supplemented with 2 g l^{-1} glucose (LBG) at 37 and 30 °C, respectively. Growth of *C. glutamicum* strains was monitored in brain–heart medium (BHI; Merck Eurolab, Darmstadt, Germany) containing 40 g l^{-1} glucose. Complex medium CGIII (Menkel et al., 1989) and minimal medium MM5 (5 g l^{-1} corn steep liquor, 20 g l^{-1} morpholinopropanesulfonic acid, 50 g l^{-1} glucose, 25 g l^{-1} $(NH_4)_2SO_4$, 0.1 g l^{-1} KH_2PO_4, 1 g l^{-1} $MgSO_4 \cdot 7H_2O$, 10 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 10 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 5 mg l^{-1} $MnSO_4 \cdot H_2O$, 0.1 g l^{-1} leucine, 0.3 mg l^{-1} biotin, 0.2 mg l^{-1} thiamin, 25 g l^{-1} $CaCO_3$) were used for the production of L-lysine with *C. glutamicum*. Antibiotics for plasmid selection were kanamycin (50 μg ml^{-1} for *E. coli* and 25 μg ml^{-1} for *C. glutamicum*) and tetracycline (5 μg ml^{-1} for *E. coli* and *C. glutamicum*).

Growth analyses with *C. glutamicum* strains were performed in a culture volume of 100 μl in a Bioscreen C Microbial Workstation (Labsystems, Vaataa, Finland). LBG overnight cultures of *C. glutamicum* were washed twice in 50 mM Tris–HCl (pH 7.5), and 2×10^5 cells were used as inoculum. The main cultures were incubated at 30 °C with intensive shaking for 48 h. The optical density (OD_{580}) was determined automatically every 2 h.

2.2. DNA isolation, manipulation and transfer

E. coli DH5αMCR (Grant et al., 1990) was used for routine cloning experiments. Vector DNA was prepared from *E. coli* cells by alkaline lysis using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). DNA restriction fragments required for cloning were purified from agarose gels by means of the QIAEX II Gel Extraction Kit (Qiagen). All recombinant DNA techniques followed standard procedures (Sambrook et al., 1989). *E. coli* and *C. glutamicum* cells were transformed by electroporation (Tauch et al., 1994, 2002b). Chromosomal DNA of *C. glutamicum* was prepared according to the method of Tauch et al. (1995).

2.3. PCR techniques

PCR experiments were carried out with a PTC-100 thermocycler from MJ Research (Watertown, MA) and *Pfu* DNA polymerase. Initial denaturation was conducted at 94 °C for 2 min followed by denaturation for 30 s, annealing for 30 s at a primer-dependent temperature, and extension at 72 °C for 45 s. This cycle was repeated 30 times, followed by a final extension step at 72 °C for 3 min. PCR products were purified using the QIA-

Table 1
Relevant *C. glutamicum* strains and plasmids used in this study

Strain or plasmid	Genetic characteristics	Source or reference
C. glutamicum		
RES167	Restriction-deficient mutant of *C. glutamicum* ATCC 13032, Δ(*cglIM-cglIR-cglIIR*)	Tauch et al., 2002b
MH1	RES167 carrying a 237-bp *Eco*RI-*Kpn*I deletion in the *dapF* gene, corresponding to amino acids 108–186	This work
MH3	RES167 carrying a 1071-bp deletion in the *dapC* gene, corresponding to amino acids 1–346	This work
MH4	RES167 carrying a 1071-bp deletion in the *dapC* gene, and a 159-bp deletion in the *ddh* gene, corresponding to amino acids 267–319	This work
MH5	RES167 carrying a 1071-bp deletion in the *dapC* gene, a 159-bp deletion in the *ddh* gene, and a 1163-bp deletion in the *argD* gene, corresponding to amino acids 1–388	This work
DSM5715	Lysine production strain, aminoethylcysteine resistant, leucine auxotroph	DSMZ[a]
MH6	DSM5715 carrying plasmid pMH10 overexpressing *dapF*	This work
MH7	DSM5715 carrying plasmid pMH12 overexpressing *dapC*	This work
Plasmids		
pCR-Blunt II	*E. coli* TOPO cloning vector	Invitrogen
pK18*mobsacB*	*E. coli* cloning vector carrying the *sacB* selection system	Schäfer et al., 1994
pMH2	pK18*mobsacB* carrying a 1.2-kb fragment with a deleted *dapF* gene	This work
pMH4	pK18*mobsacB* carrying a 1.0-kb fragment with a deleted *ddh* gene	This work
pMH6	pK18*mobsacB* carrying a 1.1-kb fragment with a deleted *dapC* gene	This work
pMH8	pK18*mobsacB* carrying a 1.1-kb fragment with a deleted *argD* gene	This work
pEC-XT99A	*E. coli-C. glutamicum* shuttle expression vector containing the IPTG inducible P_{trc} promoter	Kirchner and Tauch, 2003
pMH10	pEC-XT99A carrying *dapF* downstream of the IPTG inducible P_{trc} promoter	This work
pMH12	pEC-XT99A carrying *dapC* downstream of the IPTG inducible P_{trc} promoter	This work

[a] Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

quick PCR Purification Kit (Qiagen). Cloning of PCR products was performed in *E. coli* TOP10 by means of the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Karlsruhe, Germany).

2.4. Construction of deletion mutants of *C. glutamicum RES167*

Defined chromosomal deletions within the *dapC*, *dapF*, *ddh*, and *argD* genes of *C. glutamicum* RES167 were constructed with the pK18*mobsacB* vector system which helps to identify an allelic exchange by homologous recombination (Schäfer et al., 1994). Deletions were introduced into the respective genes by gene SOEing following the method of Horton (1995). A defined *dapF* deletion was constructed by using unique *Eco*RI and *Kpn*I restriction sites within the coding region. Defined deletions introduced into the chromosome of *C. glutamicum* were verified by PCR experiments. The length of the deleted chromosomal fragments and the corresponding amino acids of the respective proteins are given in Table 1.

2.5. Construction of *dapF* and *dapC* expression vectors

The *dapF* gene of *C. glutamicum* was amplified by PCR as 966-bp DNA fragment using *Pfu* DNA polymerase and the primer pair dapFex1 (5′-ATCGTACAATTGCACCGCACAA GCCTTGGAGA-3′) and dapFex2 (5′-GACGATGGATCCTAACGGACGAGCGCGCACTA-3′), carrying a *Mun*I (dapFex1) and a *Bam*HI (dapFex2) site within the 5′ extensions. The purified PCR product was ligated into the vector pCR-Blunt II-TOPO (Invitrogen) and the resulting plasmid was subsequently digested with *Mun*I and *Bam*HI. The *dapF* containing fragment was re-isolated from a 0.8% agarose gel and cloned into the *E. coli–C. glutamicum* shuttle expression vector pEC-XT99A (Table 1) which was previously digested with

*Eco*RI and *Bam*HI. The resulting vector used for overexpression of *dapF* in *C. glutamicum* was named pMH10.

The *dapC* gene was amplified as 1618-bp DNA fragment using primers dapCex1 (5′-GATCTAGAATTCGCCTCAGGCATAATCTAACG-3′) and dapCex2 (5′-GATCTATCTAGACAGAGGACAAGGCAATCGGA-3′), carrying an *Eco*RI (dapCex1) and an *Xba*I (dapCex2) site within the 5′ extensions. The PCR product was ligated into pCR-Blunt II-TOPO, re-isolated as *Eco*RI-*Xba*I DNA fragment from an agarose gel, and cloned into the shuttle expression vector pEC-XT99A, resulting in plasmid pMH12. The nucleotide sequences of the amplified *dapF* and *dapC* genes were verified by DNA sequencing (IIT, Bielefeld, Germany). Induction of *dapF* and *dapC* gene expression in *C. glutamicum* was carried out by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to early log-phase cultures.

2.6. Determination of epimerase activity

C. glutamicum strains were grown in minimal medium CGXII (Keilhauer et al., 1993) to determine the L,L-diaminopimelate epimerase activity. Cells were harvested after 8 h of incubation at 30 °C, washed with 20 mM Tris–HCl (pH 8.0), resuspended in buffer consisting of 20 mM Tris–HCl (pH 8.0) plus 1 mM dithiothreitol, and disrupted with a microtip-equipped sonifier. The homogenate was centrifuged for 20 min at $20\,000 \times g$ and the resulting extract was applied to a PD-10 column (Amersham-Pharmacia, Freiburg, Germany).

The activity was assayed with D,L-diaminopimelate as a substrate in a system according to the procedure of Wiseman and Nichols (1984). The assay system consisted of 200 mM Tris–HCl (pH 8.0), 40 mM hydroxylammonium chloride, 1 mM dithiothreitol, and 5 mM D,L-diaminopimelate ($>$ 90% pure). Assay mixtures were incubated at 30 °C and samples (30 μl) were taken at 0, 10, 20, and 30 min. Reactions were stopped by addition of 30 μl stop reagent (0.75 M $HClO_4$ in 7 M ethanol), neutralized with 20 μl neutralizing solution (0.1 M K_2CO_3, 20 mM Tris–HCl [pH 8.0]), and used for determination of D,D- and L,L-DAP. This was done by automated precolumn derivatization with *o*-phthaldialdehyde, separation by reversed phase chromatography (LC ChemStation HP 1900), and fluorometric detection (Jones and Gilligan, 1983). Protein concentration was determined using the method of Bensadoun and Weinstein (1976).

2.7. Determination of transaminase activity

C. glutamicum strains were grown in minimal medium MM1 (MMYE without yeast extract; Katsumata et al., 1984) to determine the *N*-succinyl-L,L-DAP aminotransaminase activity. Cells were harvested after overnight incubation at 30 °C, washed with 0.9% NaCl, resuspended in 20 mM Tris–HCl (pH 8.0), and crude extract was prepared as described before. Determination of the aminotransferase activity was based on the succinyl-DAP-dependent formation of glutamate from α-ketoglutarate. The assay system consisted of 200 mM Tris–HCl (pH 8.0), 0.25 mM PLP, 4 mM α-ketoglutarate, 8 mM *N*-succinyl-L,L-DAP, 1 mM EDTA, and gel-filtered extract. Assay mixtures were incubated at 37 °C and samples (30 μl) were taken and processed as described before.

2.8. Production of L-lysine with recombinant *C. glutamicum* strains

C. glutamicum strains were precultured in CGIII medium (Menkel et al., 1989) at 33 °C for 24 h. A main culture was inoculated in MM5 medium in such a way that the initial optical density (OD_{660}) of the culture was 0.2. After 72 h of growth at 33 °C the concentration of L-lysine in the supernatant was determined in an amino acid analyser (Eppendorf-BioTronik, Hamburg, Germany) by ion exchange chromatography and postcolumn derivatization with ninhydrin detection.

2.9. Nucleotide sequence accession numbers

The nucleotide sequences of the *dapC* and *dapF* gene loci of *C. glutamicum* ATCC 13032 were deposited in the GenBank database under accession numbers AY170830 (*dapC*) and AY170829 (*dapF*), respectively.

3. Results

3.1. Identification of the dapF gene in the whole genome sequence of C. glutamicum

The complete genome of *C. glutamicum* ATCC 13032 was recently sequenced by means of cosmid and bacterial artificial chromosome libraries (Tauch et al., 2002a). Subsequently, the genome sequence was annotated with the GenDB database system (Zentrum für Genomforschung, Bielefeld, Germany) and inspected for coding regions representing the genes involved in DAP and L-lysine biosynthesis. The already published genes of this pathway in *C. glutamicum* comprising *lysC* (*cg0306*), *asd* (*cg0307*), *dapA* (*cg2161*), *dapB* (*cg2163*), *ddh* (*cg2900*), *dapD* (*cg1256*), *dapE* (*cg1260*), and *lysA* (*cg1334*) were easily identified. In addition, a candidate gene for a putative *dapF* function (*cg2129*) was predicted. The deduced gene product of *cg2129* revealed 27% amino acid sequence identity to the DapF protein of *E. coli* (Richaud and Printz, 1988) and 32% identity to the corresponding protein of *Haemophilus influenzae* (Cirilli et al., 1998) which is the best characterized member of the diaminopimelate epimerase family. Furthermore, the *cg2129* protein showed significant amino acid sequence similarity to a large number of proteins in databases which were annotated as DapF without experimental confirmation. Especially, corresponding proteins of the taxonomically closely related species *M. tuberculosis* (52%) and *S. coelicolor* (43%) revealed high levels of amino acid sequence identity.

A PROSITE motif search within the deduced amino acid sequence of *cg2129* identified the presence of a diaminopimelate epimerase signature (PROSITE PDOC01029) comprising amino acid residues 75–89 (Fig. 2). In addition, computational secondary structure predictions (Baldi et al., 1999) for the *cg2129* protein revealed a structure of two homologous domains (amino acids 1–135 and amino acids 149–277) each containing eight β-strands and two α-helices. This structure prediction is in accordance with the three-dimensional structure of the DAP epimerase from *H. influenzae* (Cirilli et al., 1998). Two conserved cysteines, one in each domain, are involved in the catalytic

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                                         ▼
C. glutamicum   DapF   (75) NADGSLAKMCGNGVR (89)
M. tuberculosis DapF   (78) NADGSAAQMCGNGVR (92)
S. coelicolor   DapF   (76) NGDGSVAKMCGNGVR (90)
H. influenzae   DapF   (64) NADGSEVSQCGNGAR (78)
                            * ***    **** *
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Fig. 2. Diaminopimelate epimerase signature in DapF proteins. A multiple alignment of DapF protein sequences was performed with the CLUSTALW program (Thompson et al., 1994). Only the amino acid sequence of the diaminopimelate epimerase signature NxDGSx$_4$CGN[GA]xR (PROSITE PDOC01029) is shown. Conserved amino acid residues are indicated by asterisks. The conserved cysteine residue within the diaminopimelate epimerase signature is specifically marked by an arrow. Numbers in parenthesis correspond to the position of the motif with respect to the start of each protein. Protein sequences were from GenBank: *M. tuberculosis* (NC_000962), *S. coelicolor* (NC_003888), and *H. influenzae* (NC_000907).

mechanism of the DAP epimerase reaction. These conserved cysteines are also present in the deduced amino acid sequence of *cg2129* (Cys_{84} and Cys_{222}) and the active site cysteine Cys_{84} is part of the diaminopimelate epimerase signature (Fig. 2). These data strongly suggested that the *cg2129* protein is a member of the diaminopimelate epimerase family. Therefore, we have designated the *cg2129* coding region of the *C. glutamicum* genome sequence as *dapF* gene.

3.2. The C. glutamicum dapF gene is indispensable for the succinylase branch of the diaminopimelate biosynthesis

To further analyze the identified *dapF* gene, we established a 237-bp *Eco*RI-*Kpn*I deletion in the chromosomal *dapF* coding region of *C. glutamicum* RES167 by means of the *sacB* selection system present on plasmid pMH2 (Table 1). After electrotransformation into *C. glutamicum* of a recombinant *sacB* vector carrying a deletion construct, the vector can establish itself only by integration into the chromosome via homologous recombination. The resulting strain generally carries the modified gene and the wild type gene separated by the vector sequence. Excision of the vector can be selected for by growing the cells on LBG agar containing 10% sucrose. Cells able to

grow on this medium have lost the plasmid due to a second cross-over event that either restores the wild type situation or leads to a defined mutant strain (Schäfer et al., 1994). Interestingly, recombinant *C. glutamicum* strains carrying the 237-bp chromosomal *dapF* deletion were only selected for on medium (LBG+10% sucrose) which was additionally supplemented with 50 mM ammonium sulfate. Without ammonium sulfate addition only revertants to the wild type gene arrangement were obtained, indicating that the selection of a *dapF* mutant depended on the ammonium availability. The isolated *dapF* mutant strain *C. glutamicum* MH1 was, therefore, used to analyze its growth characteristics in detail.

C. glutamicum MH1 showed no phenotypic alteration during growth in standard rich media (LBG or BHI) or in minimal medium MM1 when compared with the control strain *C. glutamicum* RES167 (data not shown). On the other hand, *C. glutamicum* MH1 showed an impaired growth when supplied with low ammonium but high concentrations of carbon (BHI+4% glucose). Under this condition the growth of *C. glutamicum* MH1 arrested at an obviously lower cell density than the control strain *C. glutamicum* RES167 (Fig. 3A, left). This phenotype was initially described for *C. glutamicum* strains lacking a functional *dapD* or *dapE* gene, which can, therefore, synthesize D,L-DAP only via the dehydrogenase branch of the DAP biosynthesis pathway (Wehrmann et al., 1998). The observed growth effect was nullified by supplementation of the growth medium with 50 mM ammonium sulfate (Fig. 3A, right). In supplemented medium, *C. glutamicum* MH1 showed a growth kinetic comparable to that of the control strain *C. glutamicum* RES167, indicating that the dehydrogenase variant obviously can compensate the loss of the epimerase activity when free ammonium is available in a sufficient high concentration (Wehrmann et al., 1998). This result also provided an explanation for the necessity to supplement the growth medium with ammonium sulfate during the *sacB* selection procedure which was performed to obtain the *C. glutamicum* Δ*dapF* mutant strain MH1.

In a further genetic approach, we tried to establish an additional deletion within the *ddh* gene of the Δ*dapF* mutant strain *C. glutamicum* MH1. The recombinant *sacB* vector carrying the *ddh* deletion construct (pMH4) was integrated into the chromosome of *C. glutamicum* MH1, but it was impossible to obtain a deletion in both genes by *sacB* selection. The reason might be that *C. glutamicum* is apparently unable to take up D,L-DAP (Yeh et al., 1988), thus preventing the construction of the double mutant. This observation implies that besides the succinylase branch and the dehydrogenase variant no further pathway exists for D,L-DAP synthesis in *C. glutamicum*.

3.3. Overexpression of the dapF gene in C. glutamicum DSM5715 resulted in an increased diaminopimelate epimerase activity

In order to verify the postulated function of the *cg2129* protein as diaminopimelate epimerase, enzyme assays were performed (Table 2). Based on the rate of D,L-DAP conversion to L,L-DAP in the respective assays, a specific DapF activity of 0.01 μmol min^{-1} per mg of protein was determined in a control strain carrying the chromosomally encoded *dapF* gene, whereas no specific DapF activity was detectable in extracts of the defined *dapF* deletion mutant *C. glutamicum* MH1. In addition, the *dapF* gene was overexpressed by cloning its promotor-less coding region into the expression vector pEC-XT99A, resulting in plasmid pMH10 (Table 1). In such a way, the *dapF* gene was under control of the inducible P_{trc} promoter and present in approximately 30 copies per *C. glutamicum* chromosome (Nesvera et al., 1997). Plasmid pMH10 was transferred into the lysine-producing strain *C. glutamicum* DSM5715 and the recombinant derivative *C. glutamicum* MH6 was subsequently assayed for diaminopimelate epimerase activity. *C. glutamicum* MH6 revealed an almost 8-fold increase in the epimerase activity when compared with *C. glutamicum* DSM5715 carrying vector pEC-XT99A (Table 2). Consequently, the enzyme assays confirmed that the *cg2129* coding region of *C. glutamicum* codes for the DapF protein.

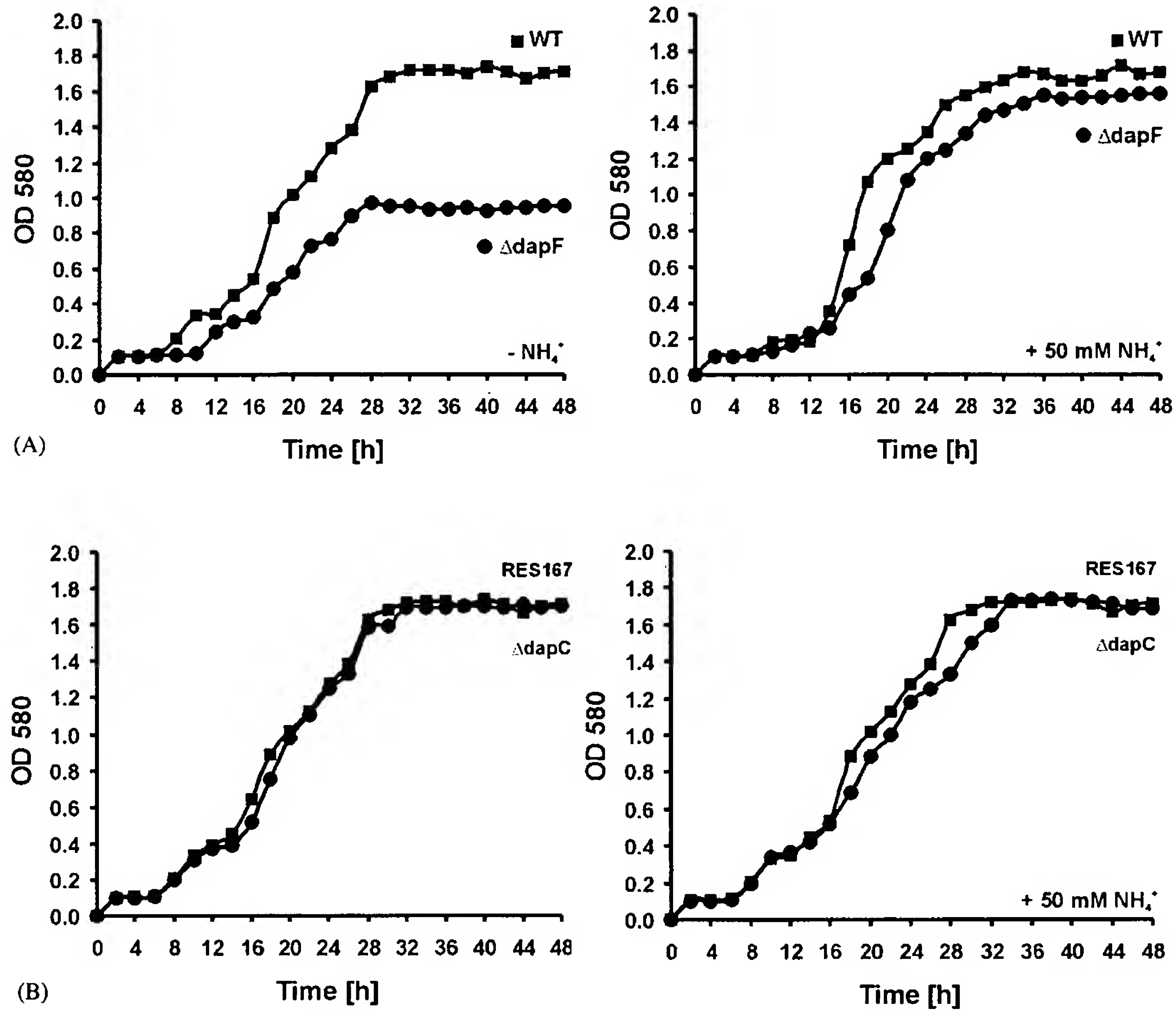

Fig. 3. Growth of *C. glutamicum* strains carrying a chromosomal *dapF* or *dapC* deletion. The growth analyses were performed in a culture volume of 100 μl in a Bioscreen C Microbial Workstation. The optical density (OD_{580}) was determined automatically. (A) Growth of the Δ*dapF* mutant *C. glutamicum* MH1 (●) was compared with *C. glutamicum* RES167 (■) in BHI+4% glucose without ammonium supplementation (left panel) and with 50 mM ammonium sulfate (right panel). (B) Growth of the Δ*dapC* mutant *C. glutamicum* MH3 (●) was compared with the control strain *C. glutamicum* RES167 (■) in BHI+4% glucose with limited ammonium availability (left panel) and with 50 mM ammonium sulfate (right panel).

Table 2
Specific diaminopimelate epimerase activity of *C. glutamicum* strains

C. glutamicum strain	*dapF* genotype	Specific activity (μmol min^{-1} per mg of protein)
DSM5715 [pEC-XT99A]	Wild type *dapF*	0.011
MH1	Deleted *dapF*	<0.001
MH6	Overexpressed *dapF*	0.087

3.4. Overexpression of the dapF gene in C. glutamicum DSM5715 led to increased L-lysine production

To analyze whether the *dapF* gene promotes a positive effect on the fermentative production of L-lysine, *C. glutamicum* MH6 was grown in MM5 medium for 72 h and the concentration of L-lysine in the culture supernatant was determined. Overexpression of the *dapF* gene increased the L-lysine concentration to 13.5 g l^{-1}, whereas the control strain (*C. glutamicum* DSM5715 containing pEC-XT99A) produced only 11.9 g l^{-1}. These values

correspond to an increase of 13.4% L-lysine within 72 h of fermentation. Therefore, the *dapF* gene is an attractive target for further improvement of lysine-producing *C. glutamicum* strains by molecular genetic engineering.

3.5. Identification of the dapC gene of C. glutamicum encoding a protein with N-succinyl-aminoketopimelate aminotransferase activity

Besides the already identified *dapF* gene, the *C. glutamicum* genome sequencing project revealed one more interesting coding region (*cg1253*) in the context of L-lysine and D,L-DAP biosynthesis in this organism. The deduced *cg1253* protein contains a conserved PLP-binding site (amino acids 224–237) and shares 29% identical amino acids with the *N*-succinyl-aminoketopimelate aminotransferase DapC of *B. pertussis*, which is the only enzymatically characterized example of DapC proteins (Fuchs et al., 2000), and 52–61% identical amino acids with putative aminotransferases from *M. tuberculosis* and *S. coelicolor*. Furthermore, *cg1253* is of particular interest as it is located in close vicinity to the *dapD* and *dapE* genes in the genome of *C. glutamicum*. In *B. pertussis* the *dapCDE* genes are located in one locus and appear to constitute an operon in this organism (Fuchs et al., 2000). The vicinity of *cg1253* to *dapD* (*cg1256*) and *dapE* (*cg1260*) in *C. glutamicum* might indicate an involvement of *cg1253* in the DAP biosynthesis pathway. The *cg1253* gene of *C. glutamicum* was, therefore, tentatively named *dapC*.

To verify the proposed *dapC* function of *cg1253*, an enzyme assay for determination of *N*-succinyl-aminoketopimelate aminotransferase activity was performed. For this purpose, we constructed a defined deletion in the *dapC* gene of *C. glutamicum* RES167 using plasmid pMH6 (Table 1). The resulting deletion mutant *C. glutamicum* MH3 was used in the assay along with the control strain *C. glutamicum* RES167 (Table 3). Based on the amount of protein used in the respective assay of *C. glutamicum* RES167, a specific DapC activity of 0.113 μmol min^{-1} per mg of protein was determined. In contrast to the control strain, no DapC activity was detectable in extracts of *C. glutamicum* MH3 carrying the *dapC* deletion. In addition, the *dapC* gene was overexpressed by cloning its promoter-less coding region into the expression vector pEC-XT99A. The resulting plasmid pMH12 (Table 1) was transferred into the lysine-producing strain *C. glutamicum* DSM5715 and the recombinant derivative *C. glutamicum* MH7 was subsequently assayed for aminotransferase activity. Compared with *C. glutamicum* DSM5715 carrying the empty vector pEC-XT99A, *C. glutamicum* MH7 revealed an almost 9-fold increase in succinyl-DAP-dependent L-glutamate accumulation (Table 3). In conclusion, both enzyme assays confirmed unequivocally that the *cg1253* coding region of the *C. glutamicum* genome codes for a protein with *N*-succinyl-aminoketopimelate aminotransferase activity.

Table 3
Specific *N*-succinyl-aminoketopimelate transaminase activity of *C. glutamicum* strains

C. glutamicum strain	*dapC* genotype	Specific activity (μmol min^{-1} per mg of protein)
RES167	Wild type *dapC*	0.113
MH3	Deleted *dapC*	< 0.001
DSM5715 [pEC-XT99A]	Wild type *dapC*	0.111
MH7	Overexpressed *dapC*	1.020

3.6. Overexpression of the C. glutamicum dapC gene led to improved L-lysine production

To analyze whether genetic engineering of the *dapC* gene can positively influence fermentative L-lysine production, *C. glutamicum* MH7 was used in a production assay. The L-lysine concentration in the supernatant of a *C. glutamicum* MH7 culture was determined after growth in MM5 medium for 72 h. Overexpression of *dapC* in *C. glutamicum* MH7 increased the L-lysine concentration to 14.7 g l^{-1}, whereas the control strain (*C. glutamicum* DSM5715 carrying pEC-XT99A) produced only 13.7 g l^{-1}, corresponding to an increase of 7.3%. Therefore, the *dapC* gene obviously represents a further interesting target for strain development in *C. glutamicum*.

3.7. The dapC gene is dispensable for the synthesis of D,L-diaminopimelate via the succinylase branch

For a further characterization of the properties of DapC in the D,L-DAP biosynthesis pathway, we tested the ability of the Δ*dapC* mutant strain *C. glutamicum* MH3 to grow on different media. As expected, *C. glutamicum* MH3 showed no phenotypic alterations when grown in complex media (LBG or BHI) or in minimal medium MM1 (data not shown). Surprisingly, there was also no growth reduction of *C. glutamicum* MH3 on rich medium (BHI+4% glucose) with excess carbon and limited ammonium availability (Fig. 3B, left). However, a reduced growth of the Δ*dapC* mutant *C. glutamicum* MH3 was expected under this growth condition, since a non-functional *dapD*, *dapE* (Wehrmann et al., 1998) or *dapF* gene (this work) was shown to cause this phenotype.

To explore this finding, we transformed *C. glutamicum* strain MH3, carrying a defined deletion in the *dapC* gene, with plasmid pMH4 (Table 1). Clones with integrated plasmid pMH4 were subsequently selected for a deleted *ddh* gene by means of the *sacB* marker system. PCR experiments confirmed that deletions in the *dapC* and *ddh* genes were present in the chromosome of *C. glutamicum* MH4. Since both branches of the D,L-DAP and L-lysine biosynthesis would be interrupted in this case, this genotype was expected to be lethal for *C. glutamicum* cells as it was already concluded from the experiment to delete the *dapF* and *ddh* gene. Therefore, it can be assumed that at least one additional enzyme is encoded in the *C. glutamicum* genome, which is able to catalyze the transamination of *N*-succinyl-aminoketopimelate to *N*-succinyl-diaminopimelate thus providing sufficient amounts of D,L-DAP for normal growth of Δ*dapC*-Δ*ddh* cells, such as *C. glutamicum* MH4. The expected activity appears to be either too low for a detection in the enzyme assay or is not detectable with the applied test system at all.

In a further experimental approach we examined whether the acetylornithine transaminase ArgD is able to substitute for the DapC function in *C. glutamicum*. It was considered as a potential candidate for a DapC bypass-reaction since a corresponding enzymatic function was described for the ArgD protein in *E. coli* (Ledwidge and Blanchard, 1999). The *E. coli* ArgD protein possesses *N*-acetylornithine and *N*-succinyl-aminoketopimelate transaminase activities and exhibits a similar catalytic efficiency for both substrates. Starting from *C. glutamicum* strain MH4, carrying deletions in the *dapC* and *ddh* gene, we constructed an additional deletion in the *argD* coding region (*cg1583*) by applying plasmid pMH8 (Table 1). After analyzing selected *C. glutamicum* mutants by PCR experiments (Fig. 4), it became evident that it was possible to delete the *dapC*, *ddh* and *argD* genes in a single *C. glutamicum* strain which was designated MH5 (Table 1). This set of defined mutations did not affect the viability of *C. glutamicum* MH5 on standard rich medium indicating that the ArgD protein is not, or not alone, capable to substitute for DapC activity. Accordingly, another yet unknown enzyme present in *C. glutamicum* must be capable to catalyze the transamination reaction in the D,L-DAP and L-lysine biosynthesis pathway in addition to the enzymatically characterized DapC protein.

4. Discussion

During decades of industrial strain design, *C. glutamicum* L-lysine producers were classically engineered by random mutagenesis and subsequent selection for desired abilities (Leuchtenberger, 1996). Nowadays, *C. glutamicum* strains with overexpressed pyruvate carboxylase gene *pyc* (Peters-Wendisch et al., 2001), mutated *lysC* (Kalinowski et al., 1990; Onishi et al., 2002) or overexpressed *dapA* gene (de Graaf et al., 2001) are examples for lysine-producing strains which were obtained by genetic engineering techniques. Identification of the lysine exporter of *C. glutamicum* provided another interesting target to improve amino acid production (Vrljic et al., 1996). Furthermore, genetic work focussed on gene targets encoding functions in the central metabolism of *C. glutamicum* (reviewed in Sahm et al., 2000), but it became more and more difficult to identify suitable target genes and to achieve further strain improvements. A new perspective

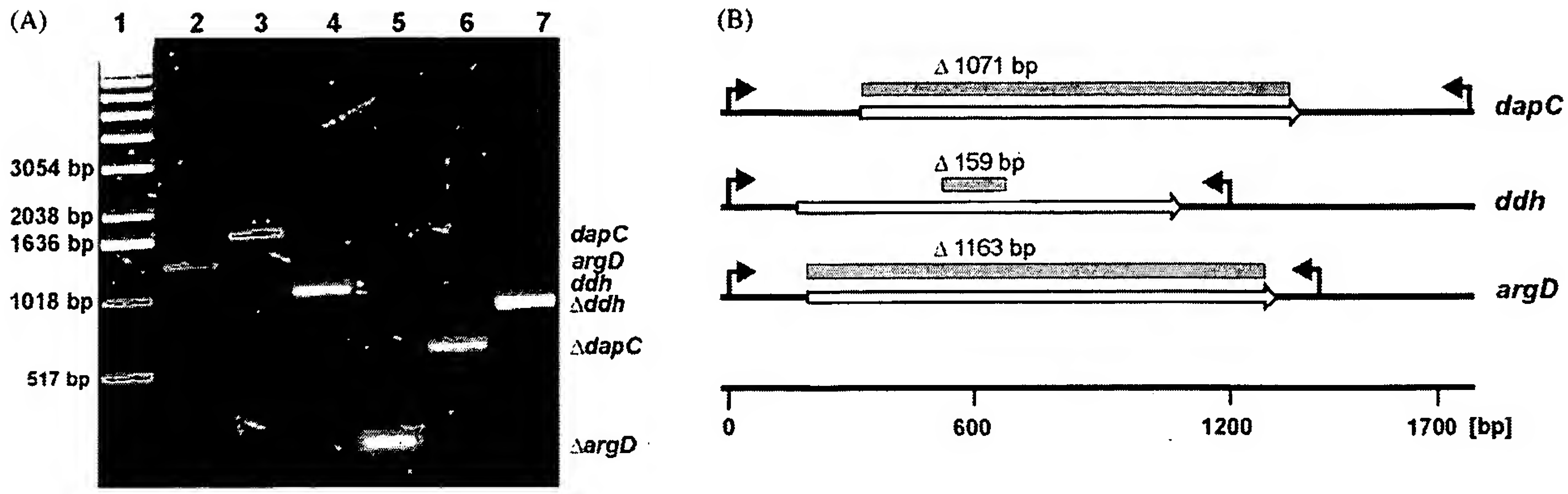

Fig. 4. Experimental confirmation of the deletions introduced into the *dapC*, *ddh* and *argD* gene of mutant strain *C. glutamicum* MH5. (A) PCR amplification of the *dapC*, *argD* and *ddh* coding regions of *C. glutamicum* RES167 (control) and of *C. glutamicum* MH5. PCR products were obtained with primer pairs located outside of each coding region. Lane 1: DNA marker X (Roche Diagnostics, Mannheim, Germany); lane 2: *argD* in RES167; lane 3: *dapC* in RES167; lane 4: *ddh* in RES167; lane 5: Δ*argD* in MH5; lane 6: Δ*dapC* in MH5; lane 7: Δ*ddh* in MH5. (B) Schematic illustration of chromosomal deletions in *C. glutamicum* MH5 and location of primers used for experimental confirmation. Approximate primer positions are indicated by arrows. Open arrows represent the corresponding genes in the control strain *C. glutamicum* RES167, grey bars symbolize the deleted regions (Δ) in *C. glutamicum* MH5. The length of the deleted DNA region is given in base pairs. The deletion within the *ddh* gene comprehends amino acid residues of the Ddh protein, which were a substantial part of the dimerization domain and the substrate-binding domain of this enzyme (Scapin et al., 1996).

on *C. glutamicum* genetic engineering was obtained by establishing the complete genome sequence of the type strain ATCC 13032 (Tauch et al., 2002a). Genome sequencing projects provide the complete genetic information of the investigated organisms and allow along with new bioinformatics tools the identification of so far not recognized target genes for genetic engineering (Hodgson, 1998). Annotation of the complete genome sequence of *C. glutamicum* led to the identification of two candidate coding regions for the last unknown genes, *dapC* and *dapF*, involved in the branched pathway for D,L-DAP and L-lysine biosynthesis of this organism. Genetic and enzymatic analyses enabled us to verify the postulated function in both cases. Cloning and overexpression of *dapC* and *dapF* resulted in increased L-lysine production in *C. glutamicum* showing that the knowledge of the complete biochemical pathway for L-lysine biosynthesis is of great relevance for biotechnological strain design.

A *C. glutamicum* mutant strain carrying a deleted *dapF* gene (MH1) showed the expected phenotype of significantly reduced growth on complex medium with high carbon content and limited availability of ammonium ions. A corresponding phenotype was initially shown for strains lacking a functional *dapD* or *dapE* gene (Wehrmann et al., 1998). These mutant strains can synthesize the essential cell wall compound D,L-DAP only via the one-step dehydrogenase branch of the pathway. The diaminopimelate dehydrogenase has a low affinity towards ammonium ions which are directly used for the reductive conversion of THDP to D,L-DAP (Misono and Soda, 1980). For *C. glutamicum* it has been shown in earlier examinations that the dehydrogenase variant is not efficiently used at an external ammonium concentration below 38 mM (Sonntag et al., 1993). This indicates that the L-lysine biosynthesis pathway is directly influenced by the free ammonium availability, probably via the kinetic characteristics of the Ddh protein. Consistent with this view is the reduced growth of *C. glutamicum* strains lacking an active succinylase variant of the pathway, as it is the case with a *dapD*, *dapE* or a *dapF* mutant.

Surprisingly, *C. glutamicum* MH3, carrying a defined *dapC* deletion, did not show the characteristic growth behavior of mutant strains with a non-functional succinylase branch. Even a simultaneous deletion of the *dapC* gene together with

the *ddh* gene could be established in *C. glutamicum*. This is a strong evidence that *C. glutamicum* possesses at least one additional aminotransferase, which is able to substitute for the mutated *dapC* gene function of *C. glutamicum* MH3. In *E. coli*, the ArgD protein fulfills transamination reactions in both the arginine and D,L-DAP biosynthesis pathways (Ledwidge and Blanchard, 1999). However, growth analyses of *C. glutamicum* MH5, deleted in the *dapC*, *ddh*, and *argD* gene, indicates that another yet unknown enzyme is able to substitute the DapC function in *C. glutamicum*. To further investigate this phenomenon, the *C. glutamicum* genome sequencing project provides a very promising basis, because it allows a systematic approach for analyzing the entirety of aminotransferases encoded in the chromosome. In conclusion, the *C. glutamicum* genome sequencing project provided great benefit with regard to the identification of potential target genes useful for industrial strain design. The identification of the *dapC* and *dapF* gene in *C. glutamicum,* described here, is considered to be an impressive example of the application of genome research for strain optimization.

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